METHOD DEVELOPMENT AND VALIDATION FOR THE SIMULTANEOUS ESTIMATION OF EZETIMIBE AND GLIMEPIRIDE BY USING RP –HPLC AND UV METHOD

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ABSTRACT

The number of drugs introduced into the market is increasing every year. These drugs may be either new entities or partial structural modification of the existing one. Often a time lag exists from the date of introduction of a drug into the market to the date of its inclusion in pharmacopoeias. Hence, standards and analytical procedures for these drugs may not be available in the pharmacopoeias. It becomes necessary, therefore to develop newer analytical methods for such drugs. Hence the present research work was designed to develop a new RP HPLC method for the simultaneous estimation of Ezetimibe and Glimepiride in pharmaceutical dosage form. The wavelength of maximum absorption (λ_{max}) of the drug, 10 μg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The Isosbestic point was found to be 228 nm for Ezetimibe and Glimipride combination. Efficiency was more and resolution was good with mobile phase Triethylamine: Methanol (30:70) at 228 nm. Asymmetry was good. Hence the method was optimized. The amount of Ezetimib and Glimipride present in the taken dosage form was found to be 99.94% and 99.89% respectvely.

Key words: Ezetimibe, Glimepiride, RP HPLC, simultaneous estimation, Isosbestic point.

INTRODUCTION

High-performance liquid chromatography (or High pressure liquid chromatography, HPLC) is a Specific form of column chromatography generally used in biochemistry and analysis to separate, identify, and quantify the active compounds. [1] HPLC mainly utilizes a column that holds packing material (stationary phase), a pump that moves the mobile phase(s)

through the column, and a detector that shows the retention times of the molecules. Retention time varies depending on the interactions between the stationary phase, the molecules being analyzed, and the solvent(s) used. [2] The sample to be analyzed is introduced in small volume to the stream of mobile phase and is retarded by specific chemical or physical interactions with the stationary phase. The amount of retardation depends on the nature of the analyte and composition of both stationary and mobile phase. The time at which a specific analyte elutes (comes out of the end of the column) is called the retention time. Common solvents used include any miscible combinations of water or organic liquids (the most common are methanol and acetonitrile). [2, 3] Sepration has been done to vary the mobile phase composition during the analysis; this is known as gradient elution. [3] The gradient separates the analyte mixtures as a function of the affinity of the analyte for the current mobile phase. The choice of solvents, additives and gradient depend on the nature of the stationary phase and the analyte.

TYPES OF HPLC

Types of HPLC generally depend on phase system used in the process. [3, 4] Following types of HPLC generally used in analysis-

Normal phase chromatography: Also known Normal phase HPLC (NP-HPLC), this method separates analytes based on polarity. NP-HPLC uses a polar stationary phase and a non-polar mobile phase. The polar analyte interacted with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase increases the elution time.

Reversed phase chromatography: Reversed phase HPLC (RP-HPLC or RPC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. RPC operates on the principle of hydrophobic interactions, which result from repulsive forces between a polar eluent, the relatively non-polar analyte, and the non-polar stationary phase. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand in the aqueous eluent.

Size exclusion chromatography: Size exclusion chromatography (SEC), also called as gel permeation chromatography or gel filtration chromatography mainly separates particles on the basis of size. It is also useful for determining the tertiary structure and quaternary structure of proteins and amino acids. This technique is widely used for the molecular weight determination of polysaccharides.

Ion exchange chromatography: In Ion-exchange chromatography, retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. This form of chromatography is widely used in purifying water, Ligand-exchange chromatography, Ion-exchange chromatography of proteins, High-pH anion-exchange chromatography of carbohydrates and oligosaccharides, etc. [3, 4]

Bio-affinity chromatography: Separation based on specific reversible interaction of proteins with ligands. Ligands are covalently attached to solid support on a bio-affinity matrix, retains proteins with interaction to the column-bound ligands.

Proteins bound to a bioaffinity column can be eluted in two ways:

- Biospecific elution: inclusion of free ligand in elution buffer which competes with column bound ligand.
- Aspecific elution: change in pH, salt, etc. which weakens interaction protein with column-bound substrate.

Because of specificity of the interaction, bioaffinity chromatography can result in very high purification in a single step (10 - 1000-fold).

PARAMETERS

For the accurate analysis of a compound, there are some parameters which are used as a standard for a particular compound. If there is a change occurs in the parameters the result may be affected greatly. The most commonly used parameters are internal diameter, particle size, pore size, pump pressure. For different compounds the parameters can be changed according to their nature and chemical properties.

Internal diameter: The internal diameter (ID) of an HPLC column is a critical aspect that determines quantity of analyte that can be loaded onto the column and also influences sensitivity. Larger columns are usually seen in industrial applications such as the purification of a drug product for later use. Low ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

Particle size: Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). Smaller particles generally provide more surface area and better separations, but the pressure required for optimum linear velocity increases by the inverse of the particle diameter squared.

Pore size: Many stationary phases are porous to provide greater surface area Small pores provide greater surface area while larger pore size has better kinetics especially for larger analytes. Pore size defines an ability of the analyte molecules to penetrate inside the particle

and interact with its inner surface. This is especially important because the ratio of the outer particle surface to its inner one is about 1:1000. The surface molecular interaction mainly occurs on the inner particle surface. [5]

Pump pressure: Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Modern HPLC systems have been improved to work at much higher pressures, and therefore be able to use much smaller particle sizes in the columns (< 2 micrometres).

Method development involves considerable trial and error procedures. Analytical methods for a drug in combination with other drugs may not be available. The existing analytical procedures may require expensive reagents and solvents. [6] It may also involve cumbersome extraction and separation procedures and these may not be reliable.

The most difficult problem usually is where to start, what type of column is worth trying with what kind of mobile phase.[7,8,9]

Hence the present research was designed to develop new RP HPLC method for the simultaneous estimation of Ezetimibe and Glimepiride in pharmaceutical dosage form.

MATERIALS AND METHODS

Determination of Working Wavelength (λmax)

In simultaneous estimation of drugs wavelength maxima is used. So this wavelength is used in estimation to estimate drugs accurately.

Preparation of standard stock solution of Ezetimibe:

10mg of Ezetimibe was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Preparation of standard stock solution of Glimipride [10]:

10mg of Glimipride was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

Method Development of Ezetimibe and Glimepiride

Chromatographic conditions

- Mobile phase : Triethylamine: Methanol
- Ratio : 30:70
- Column : Zodiac, C18 (250×4.6× 5µ)

- Wavelength : 228nm
- Flow rate : 1ml/min
- pH :4.0

Preparation of standard stock solution:

Weigh accurately 100mg of Ezetimibe and 10mg of Glimepiride in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase From above stock solution 100µg/ml of Ezetimibe and 10 µg/ml of Glimepiride is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Optimized chromatographic conditions:

Mobile phase	Triethylamine: Methanol (30:70)
pH	4.0
Column	INERTSIL column,C18(150x4.6 ID) 5µm
Flow rate	1.0 ml/min
Column temperature	Room temperature(20-25°C)
Sample temperature	Room temperature(20-25°C)
Wavelength	228nm
Injection volume	20 µl
Run time	8min
Retention time	About 3.317min for Ezetimibe and min for Glimipride

Preparation of samples for Assay

Preparation of mixed standard solution: Weigh accurately 100mg of Ezetimibe and 10mg of Glimepiride in 100 ml of volumetric flask and dissolve in 10ml of mobile phase and make up the volume with mobile phase From above stock solution 100µg/ml of Ezetimibe and 10 µg/ml of Glimepiride is prepared by diluting 1ml to 10ml with mobile phase. This solution is used for recording chromatogram.

Preparation of sample solution: 5tablets (each tablet contains 10mg of Ezetimibe and 1mg of Glimipride) were weighed and taken into a mortar and crushed to fine powder and

uniformly mixed. Tablet stock solutions of Ezetimibe (100µg/ml) and Glimepiride (10µg/ml) were prepared by dissolving weight equivalent to 100mg of Ezetimibe and 10 mg of Glimipride and dissolved in sufficient mobile phase. After that filtered the solution using 0.45-micron syringe filter and Sonicated for 5 min and dilute to 100ml with mobile phase. Further dilutions are prepared in 5 replicates of 100µg/ml of Ezetimibe and 10 µg/ml of Glimipride was made by adding 1ml of stock solution to 10 ml of mobile phase.

RESULTS AND DISCUSSION

Solubility Studies

These studies are carried out at 25 ^{0}C

EZETIMIB:

Freely to very soluble in ethanol, methanol, and acetone and practically insoluble in water

GLIMIPRIDE:

freely soluble in water , DMSO and Methanol.

Determination of Working Wavelength (λmax)

In simultaneous estimation of drugs wavelength maxima is used. So this wavelength is used in estimation to estimate drugs accurately.

Preparation of standard stock solution of Ezetimib:

10mg of Ezetimib was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

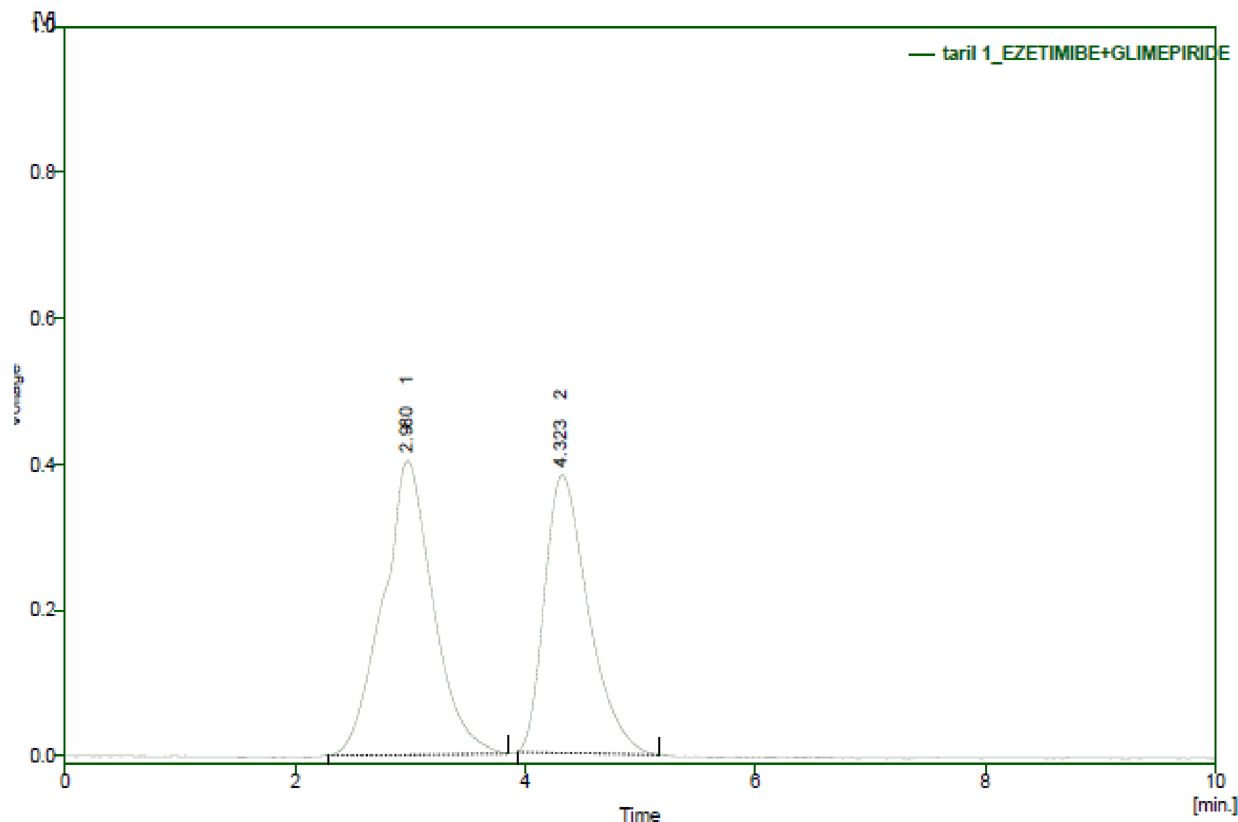
Preparation of standard stock solution of Glimipride:

10mg of Glimipride was weighed and transferred in to 100ml volumetric flask and dissolved in methanol and then make up to the mark with methanol and prepare 10 µg /ml of solution by diluting 1ml to 10ml with methanol.

The wavelength of maximum absorption (λ_{max}) of the drug, 10 µg/ml solution of the drugs in methanol were scanned using UV-Visible spectrophotometer within the wavelength region of 200–400 nm against methanol as blank. The resulting spectra are shown in the fig. no. 8.1, 8.2 and 8.3 and the absorption curve shows characteristic absorption maxima at nm for Ezetimib and nm for Glimipride nm for the combination.

Sample Info:

Sample ID	: MeOH:Phosphate buffer (75:25) PH:4	Amount	: 1
Sample	: Ezetimibe+Glimepiride	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - tarll 1_EZETIMIBE+GLIMEPIRIDE)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.980	12237.607	402.924	54.84	51.4	0.47
2	4.323	10075.477	380.698	45.16	48.6	0.41
	Total	22313.084	783.622	100.00	100.0	

Column Performance Table (From 50% - tarll 1_EZETIMIBE+GLIMEPIRIDE)

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.980	0.467	1.050	0.00	226	2259	-
2	4.323	0.407	1.767	0.00	626	6261	1.810

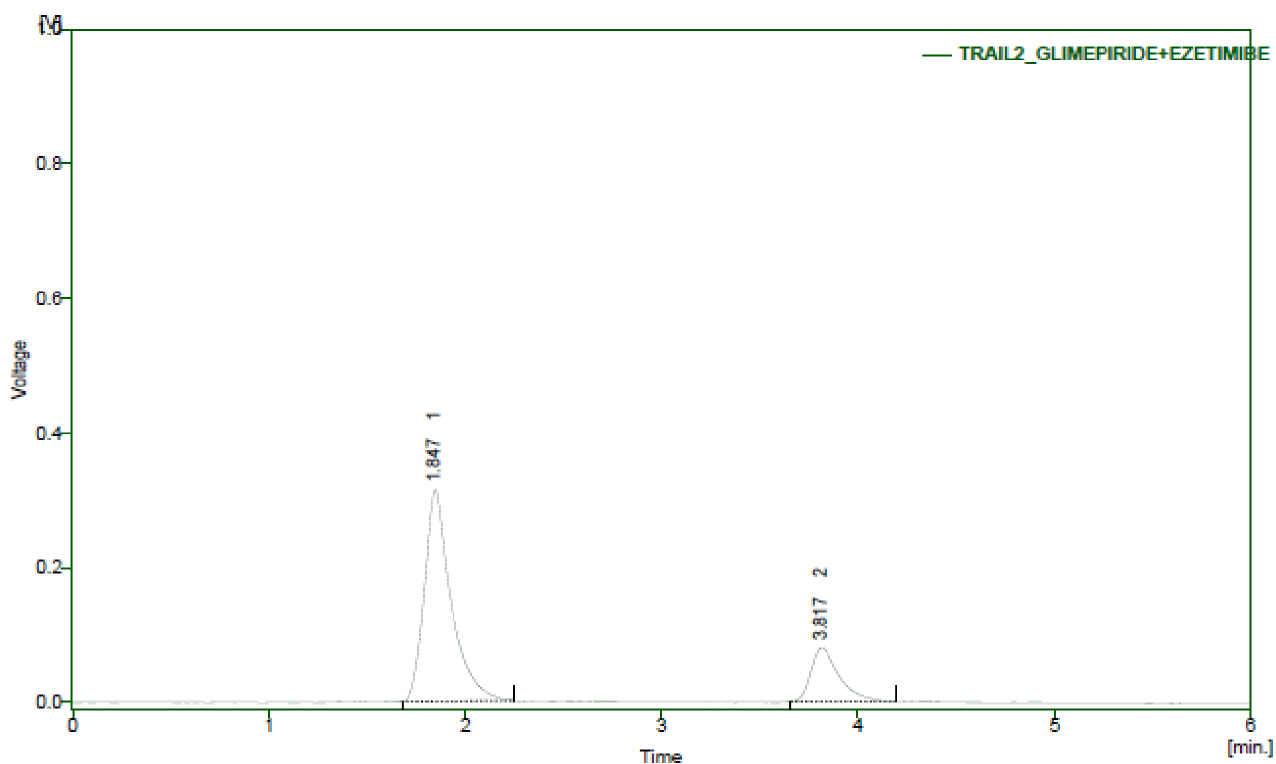
Figure 1: Chromatogram of Ezetimib and Glimipride

Observation:

Resolution was less efficiency was less.

Sample Info:

Sample ID	: Methanol:ACN:Water(70:10:20)	Amount	: 1
Sample	: Glimepiride+Ezetimibe	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - TRAIL2_GLIMEPIRIDE+EZETIMIBE)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	1.847	2965.135	314.285	78.71	79.7	0.14
2	3.817	801.815	80.147	21.29	20.3	0.15
	Total	3766.950	394.431	100.00	100.0	

Column Performance Table (From 50% - TRAIL2_GLIMEPIRIDE+EZETIMIBE)

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	1.847	0.137	1.765	0.00	1011	10115	-
2	3.817	0.150	1.969	0.00	3587	35867	8.087

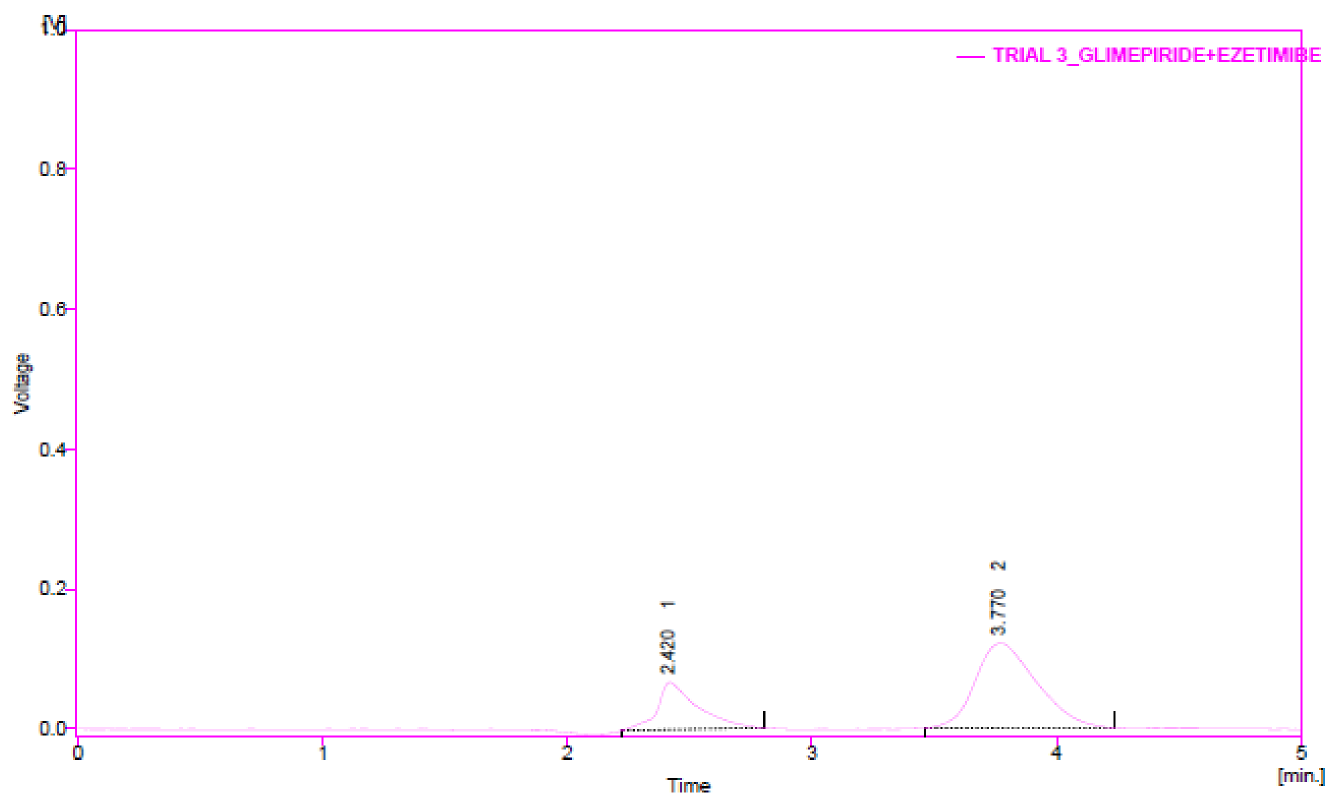
Figure 2: Chromatogram of Ezetimib and Glimipride

Observation:

Tailing was occurred and shapes of the peaks was not good.

Sample Info:

Sample ID	: Methanol:ACN:Water 75:15:10	Amount	: 1
Sample	: Glimepiride+Ezetimibe	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - TRIAL 3_GLIMEPIRIDE+EZETIMIBE)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	2.420	774.063	67.761	26.74	35.7	0.16
2	3.770	2120.372	121.973	73.26	64.3	0.27
	Total	2894.435	189.734	100.00	100.0	

Column Performance Table (From 50% - TRIAL 3_GLIMEPIRIDE+EZETIMIBE)

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	2.420	0.157	1.930	0.00	1322	13219	-
2	3.770	0.273	1.567	0.00	1054	10539	3.695

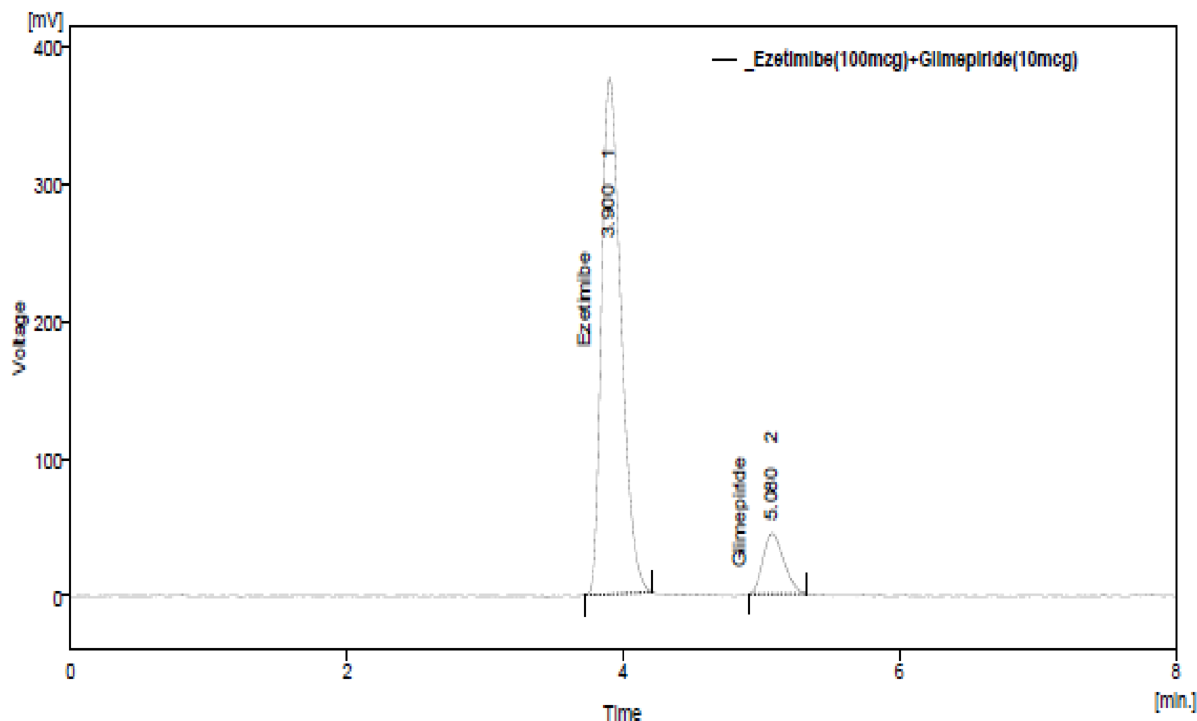
Figure 3: Chromatogram of Ezetimib and Glimipride

Observation:

Tailing and asymmetry was more and shapes of the peaks were not good.

Sample Info:

Sample ID	:optimized trail	Amount	: 1
Sample	: Ezetimibe(100mcg)+Glimepiride(10mcg)	ISTD Amount	: 0
Inj. Volume [ml]	: 0.02	Dilution	: 1



Result Table (Uncal - _Ezetimibe(100mcg)+Glimepiride(10mcg))

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]
1	3.900	3710.098	376.000	88.9	89.5
2	5.080	463.871	44.136	11.1	10.5
	Total	4173.968	420.136	100.0	100.0

Column Performance Table_Ezetimibe(100mcg)+Glimepiride(10mcg))

	Reten. Time	W05 [min]	Asymmetry [-]	Capacity [-]	Efficiency [th.pl]	Eff/l [t.p./m]	Resolution [-]
1	3.900	0.157	1.688	0.00	3433	34331	-
2	5.080	0.170	1.395	0.00	4947	49470	4.251

Figure 4: Chromatogram of Ezetimib and Glimipride

Observation:

Efficiency was more and resolution was good. Asymmetry was good. Hence the method was optimized.

A simple and selective LC method is described for the determination of Ezetimib and Glimepride dosage forms. Chromatographic separation was achieved on a c18 column using mobile phase consisting of a mixture of Triethylamine: Methanol (30:70 v/v), with detection

of 244nm. Linearity was observed in the range 60-140µg /ml for Ezetimib (r^2 =0.997) and 6-14µg /ml for Glimepride for the amount of drug estimated by the proposed methods was in good agreement with the label claim. The proposed methods were validated. The accuracy of the methods was assessed by recovery studies at three different levels. Recovery experiments indicated the absence of interference from commonly encountered pharmaceutical additives. The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. All statistical data proves validity of the methods and can be used for routine analysis of pharmaceutical dosage form.

CONCLUSION

From the above experimental results and parameters it was concluded that, this newly developed method for the simultaneous estimation of Ezetimib and Glimepride was found to be simple, precise, accurate and high resolution and shorter retention time makes this method more acceptable and cost effective and it can be effectively applied for routine analysis in research institutions, quality control department in industries, approved testing laboratories, bio-pharmaceutical and bio-equivalence studies and in clinical pharmacokinetic studies in near future.

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